ESTABLISHING A WORKING PROTOCOL FOR PLASMID CLONING AND SHRNA DESIGN IN ENDOGENOUS BRACHIONUS MANJAVACAS GENE TRP7

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ESTABLISHING A WORKING PROTOCOL FOR PLASMID CLONING AND SHRNA DESIGN IN ENDOGENOUS BRACHIONUS MANJAVACAS GENE TRP7

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LIST OF ABBREVIATIONS

RNAi	RNA interference
shRNA	short hairpin RNA
B. manjavacas	*Brachionus manjavacas*
TRP	Transient receptor potential
PCR	Polymerase Chain Reaction
ASW	Artificial Salt Water
T. suecica	*Tetraselmis suecica*
B. rotundiformis	*Brachionus rotundiformis*
B. plicatilis	*Brachionus plicatilis*
B. calyciflorus	*Brachionus calyciflorus*

SUMMARY

Current transfection protocol in rotifers only allows for temporary transfection within rotifers and does not allow for the continuous knockdown of endogenous genes, thereby inhibiting the possibility of observing long-term biological effects in response to specific perpetual gene knockdowns. This study aims to address this particular issue by establishing a working protocol for plasmid cloning and shRNA design within an endogenous gene of *B. manjavacas* with known biological effects, allowing for the exploration into the optimization of a transfection protocol and demonstration of RNAi knockdown of the known gene within the rotifers as subsequent studies. Manipulation of gene expression in rotifers could occur through plasmid vector insertions, which induce silencing of a gene's expression with short hairpin RNA (shRNA), via RNAi[1]. This would effectively stimulate gene knockdown, allowing for the observation of biological effects such as changes in fecundity and lifespan. With the establishment of a working protocol for plasmid cloning and shRNA design, as a result of this study, the optimization of a transfection protocol for rotifers is explored. With increased efficiency in the transfection of rotifers, populations of rotifers expressing the plasmid can be amassed, allowing for experimental design that examine the varying aging mechanisms and effects that are stimulated due to permanent changes in target gene expression through RNAi. This, in turn, could give rise to the identification of evolutionarily conserved genes that regulate organismal aging, which could lead to further implications in the field of pharmacological intervention in mammalian aging as well as in the field of biogerontology overall.

CHAPTER 1

INTRODUCTION

Over the past century, in general, a dramatic increase in lifespan has been observed around the globe[2]. Variability in lifespan is still present however, and it has been speculated that part of this variability is attributable to genetic origins[3]. Consequently, identifying and understanding the genetic mechanisms and pathways that extend longevity has become one of the primary goals in the field of biogerontology.

Research within the field of biogerontology, or the study of aging, generally utilizes invertebrate organisms for observation and experimentation. Invertebrate model studies have propelled research in the field of biogerontology, leading to the identification of many genes and conserved longevity pathways that have shown to regulate longevity across diverse eukaryotic species[4]. Several organisms' genomes have been explored through the use of RNA interference (RNAi) technology, in order to ascertain aging-related genes and conserved longevity pathways in their respective genomes[4]. The fundamental models for research within this field are *Drosophila melanogaster* and *Caenorhabditis elegans*. Though they have made significant contributions to the field of biogerontology, it has recently been discovered that these models have certain acute shortcomings that inhibit them from being the quintessential models for research within this field. One of the major shortcomings is the extensive gene loss that these species have undergone since their initial divergence from their common ancestor with humans[5]. This signifies that there may potentially be a substantial amount of genomic material that shares relevance to humans that has yet to be screened for aging

genes and its subsequent conserved longevity pathways. The accumulation of these shortcomings, in turn, gave rise to the speculation that exploration into additional invertebrate models for research could be a worthwhile venture[6]. One such new invertebrate model explored is the species, *Brachionus manjavacas*. *B. manjavacas*, a member of the eukaryotic, marine, monogonont rotifer species, serve as a particularly good candidate for aging studies due to their short generation times, ease of culturing, asexual proliferation of clonal cultures, and pre-existing tool box of genetic resources that include partially sequenced genomes, transcriptomes, and working RNAi protocol[7]. Additionally, *B. manjavacas* also allow for the evaluation of unique genes and pathways for their potential significance to aging genes and conserved longevity pathways overall.

There are numerous methods that can explore a specific gene's impact on longevity. In *C. elegans*, highly expressed genes were analyzed through the use of microarray methodology platforms in order to analyze their gene expression profiles. Additionally, genes were also often mutated and/or manipulated in order to observe their impact on the organism overall[8]. The latter approach is of significant interest for this study. One methodology used to manipulate the expression of genes, and thereby observe the biological impact of that gene on the organism, is through the practice of gene knockdown. Gene knockdown refers to an experimental technique where the expression of an organism's gene is inhibited or silenced due to RNA interference. This cellular mechanism entails the introduction of short hairpin RNA (shRNA) into the organismal cells of interest through transduction with plasmid vectors, allowing for both the stable integration of the shRNA as well as the long-term knockdown of the targeted gene of interest[9]. A study on honeybee workers confirmed the efficacy of the use of this

technique. Knockdown of vitellogenin, a york precursor protein found in honeybee workers, significantly increased lifespan[10].

Interest has grown in the application of this technique on rotifers in order to identify additional aging genes that these organisms may potentially share with humans. Current studies regarding manipulation of gene expression in rotifers, however, have yet to establish a transfection protocol that allows for the continuous knockdown of genes. The current protocol allows for the transfection of double stranded small interfering RNA (siRNA) into resting eggs, hatched rotifers, and amictic eggs[11]. This occurs through the uptake of the siRNA into their tissues through hydration, ingestion, and lipofection respectively[11]. This protocol, however, only allows for temporary transfection within rotifers and does not allow for the knockdown of endogenous genes, thereby inhibiting the possibility of observing biological effects in rotifers in response to specific perpetual gene knockdowns.

One way to address this particular issue is through the demonstration of RNAi knockdown in an endogenous gene of *B. manjavacas* whose function has been characterized. Delivery of plasmid DNAs, red fluorescent proteins, and semiconductor quantum dots into rotifers with the use of nonviral technology, specifically with a group of short, membrane-permeable cationic peptides called cell-penetrating peptides, have been observed[12]. Active expression of these cell-penetrating peptide delivered plasmid DNAs containing the enhanced green fluorescent protein and red fluorescent protein coding sequences was detected through the use of a function reporter gene, leading to interest in the use of viral technology for delivering nanomaterials and macromolecules into the nucleus of live rotifer cells, as per this study's proposed method for

demonstrating RNAi knockdown in an endogenous gene of *B. manjavacas* whose function has been characterized[12]. Manipulation of gene expression in rotifers could occur through plasmid vector insertions, which induce silencing of a gene's expression with shRNA, via RNAi[1]. This would effectively stimulate gene knockdown, allowing for the observation of biological effects such as changes in fecundity and lifespan.

This study aims to establish a working protocol for plasmid cloning and shRNA design within an endogenous gene of *B. manjavacas* with known biological effects, allowing for the demonstration of RNAi knockdown of the known gene within the rotifers as a subsequent study. With the establishment of a working protocol for plasmid cloning and shRNA design, optimization of the transfection protocol in rotifers can be explored. With increased efficiency in the transfection of rotifers, populations of rotifers expressing the plasmid can be amassed, allowing for experimental design that examine the varying aging mechanisms and effects that are stimulated due to permanent changes in target gene expression as a result of RNAi. This, in turn, could give rise to the identification of evolutionarily conserved genes that regulate organismal aging, which could lead to further implications in the field of pharmacological intervention in mammalian aging as well as in the field of biogerontology overall.

CHAPTER 2

METHODS

Initially, the endogenous *B. manjavacas* gene TRP7 was speculated to impact aging based on life table analysis of rotifers under moderately lower temperatures and RNAi[13]. Transient receptor potential (TRP) ion channels enact a role in thermosensation[14], and studies have found that moderately lower temperatures greatly extend the lifespan of *B. manjavacas*[13]. This makes TRP7 a good candidate for further gene manipulation studies.

This study aimed to establish a working protocol for plasmid cloning and shRNA design by designing a plasmid with shRNA for the *B. manjavacas* gene TRP7. A pCS2+ plasmid designed by the Turner Lab at the University of Michigan, was structured so as to contain the simian CMV IE94 promoter sequence. This promoter sequence is actively recognized by the endogenous rotifer RNA polymerase II[15]. Restriction sites in the plasmid allow for the insertion of shRNA within the plasmid. TRP7 shRNA oligos and PCR primers were designed. The TRP7 gene sequence was acquired from the *B. manjavacas* transcriptome, and two reverse complementary oligonucleotides were designed with an shRNA loop, using the Thermo Fisher Scientific Block-iT™ RNAi Designer tool, so as to create a 70 base pair shRNA insert specific to the TRP7 gene.

Restriction enzymes for BamHI and NcoI directly following the SP6 promoter site (Figure 1) were used to digest 1000 ng of plasmid over an incubation period of two hours at 37°C before they were denatured. The vector was then dephosphorylated with antarctic phosphatase over an incubation period of two hours at 37°C before the enzyme

were denatured at 75°C for a period of 5 minutes. Subsequently, 4.346 ng of insert was ligated into 100 ng of vector at a 1 to 3 vector to insert ratio to attain cohesive end ligations using T4 DNA ligase. This ligation reaction was incubated at 16°C for a period of 18 hours. The ligated plasmids were then transformed into *Escherichia Coli* top 10 competent cells. The original pCS2eGFP plasmid was also transformed into the competent cells as a positive control. The purpose of the control in this phase of the experiment was to establish the efficacy of the transformation protocol.

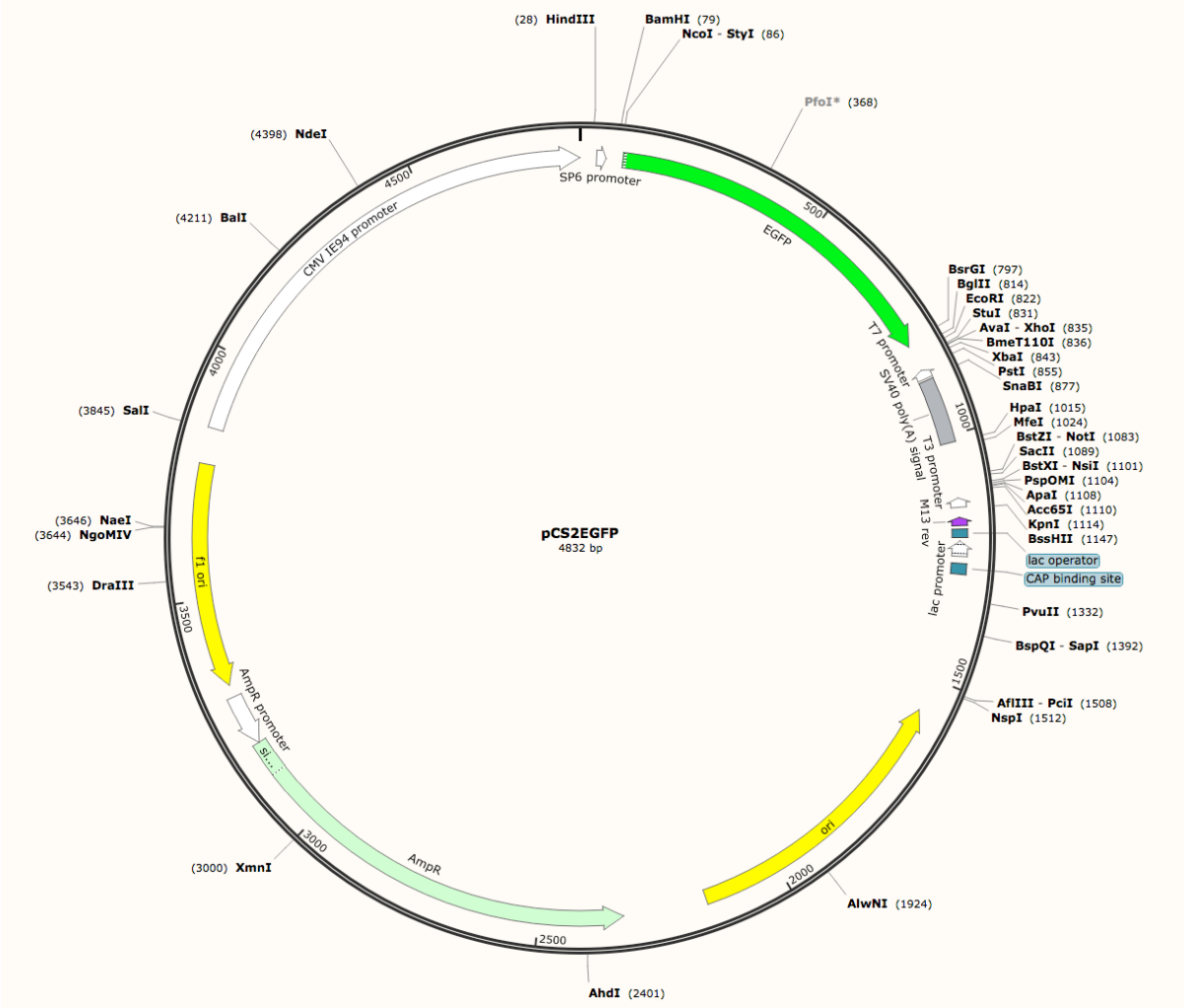

Figure 1: Unmodified pCS2eGFP plasmid

Caption: The pCS2eGFP plasmid illustrated above was the plasmid utilized to establish a working protocol for plasmid cloning and shRNA design by designing a plasmid with shRNA for the *B. manjavacas* gene TRP7. The TRP7 gene was ligated in between the BamHI and NcoI restriction sites following the SP6 promoter site.

200uL of the positive control and ligated plasmid transformed bacteria were then respectively plated on LB plates with and without Ampicillin and incubated for 24 hours at 37°C. Colony PCR using an SP6 primer and a reverse pCS2+ primer, designed from the unmodified pCS2eGFP plasmid, was then used to amplify a target region containing the first 500 base pairs and allow for the verification of the insertion of the shRNA into the plasmid. Colonies speculated to contain the shRNA insert, from Colony PCR analysis, were further verified through Sanger sequencing for confirmation of the presence of the shRNA insert within the plasmid vector.

Upon verification of successful insertion and cloning of the TRP7 shRNA construct, an optimization of the transfection protocol was explored. Animals were transfected, in mass, with the use of a polymeric transfection reagent. The overall experimental design used for transfecting the animals incorporated the use of 24-well plates, 15 PPT artificial salt water (ASW), the pCS2+ plasmid designed by the Turner Lab at the University of Michigan, the unmodified pCS2eGFP plasmid (Figure 1), a polymeric and/or nonliposomal transfection reagent, and *B. manjavacas, Brachionus rotundiformis, Brachionus plicatilis,* or *Brachionus calyciflorus* hatchlings or resting eggs. Rotifers were added to 24-well plates containing 500µL of 15 PPT ASW with/without *Tetraselmis suecica*, depending on the treatment, and an optimized solution volume of transfection reagent to DNA, containing approximately 500 ng of pCS2eGFP plasmid. Controls used nuclease free water or the pCS2+ plasmid in place of the GFP containing plasmid. The purpose of the control well in these experiments was to establish the baseline fluorescence of the rotifers utilized. The rotifers were then left to incubate over the time period of 24 hours in a 25°C, 30°C, or 35°C incubator with or without

access to light, depending on the experiment. A list of the varying transfection conditions tested is provided below (Table 1).

Table 1: Transfection experiments conducted on *B. manjavacas, B. rotundiformis, B. plicatilis,* and *B. calyciflorus*

Experiment	**Treatment**	**Period of Incubation before Animals are Imaged (hrs.)**
1	*B. manjavacas* hatchlings were transfected with Viromer® Red polymeric transfection solution and nuclease free water (control) or pCS2eGFP plasmid and incubated in an environment without light at 25°C.	24
2	*B. manjavacas* hatchlings were transfected with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment without light at 25°C.	24
3	*B. manjavacas* hatchlings transfected with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid were subsequently fed with media containing a minimal volume of *T. suecica* at a concentration of 2 x10^5 cells/mL 24 hours after transfection and incubated in an environment without light at 25°C.	48
4	*B. manjavacas* hatchlings fed with media containing a minimal volume of *T. suecica* at a concentration of 2 x10^5 cells/mL were subsequently transfected 24 hours after feeding with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment without light at 25°C.	48
5	*B. manjavacas* hatchlings transfected with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid were fed with media containing a minimal volume of *T. suecica* at a concentration of 2 x10^5 cells/mL 24 hours after transfection and transfected again with Viromer® Yellow polymeric transfection solution 48 hours after the initial transfection and incubated in an environment without light at 25°C.	96
6	*B. manjavacas* resting eggs were hydrated with	24

	Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	
7	*B. manjavacas* hatchlings were transfected with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment without light at 25°C.	24
8	*B. manjavacas* hatchlings were transfected with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment without light at 30°C.	24
9	*B. manjavacas* hatchlings were transfected with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment without light at 35°C.	24
10	*B. manjavacas* hatchlings, resultant from experiment seven's transfection over a period of 24 hours, were subsequently fed with media containing a minimal volume of *T. suecica* at a concentration of 2 x10^5 cells/mL and incubated in an environment without light at 35°C.	48
11	*B. manjavacas* resting eggs were transfected with a 0.5µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	24
12	*B. manjavacas* resting eggs were transfected with a 0.5µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°. The resultant hatchings were fed with media containing a minimal volume of *T. suecica* at a concentration of 2 x10^5 cells/mL 24 hours after transfection incubated in an environment without light at 30°C.	48
13	*B. manjavacas* resting eggs were transfected with a 1µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	24
14	*B. manjavacas* resting eggs were transfected with a 1µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°. The resultant hatchings were fed with media containing a minimal volume of *T. suecica* at a	48

	concentration of 2 x10^5 cells/mL 24 hours after transfection incubated in an environment without light at 30°C.	
15	*B. manjavacas* resting eggs were transfected with a 2µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	24
16	*B. manjavacas* resting eggs were transfected with a 1µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°. The resultant hatchings were fed with media containing a minimal volume of *T. suecica* at a concentration of 2 x10^5 cells/mL 24 hours after transfection incubated in an environment without light at 30°C.	48
17	*B. rotundiformis* resting eggs were transfected with a 0.5µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	24
18	*B. plicatilis* resting eggs were transfected with a 0.5µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	24
19	*B. calyciflorus* resting eggs were transfected with a 0.5µg concentration of Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	24
20	*B. manjavacas* resting eggs were transfected with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid for 1 hour before being transferred to a media with 500 µL 15PPT ASW and incubated in an environment with light at 25°C.	24
21	*B. manjavacas* resting eggs were transfected with Fugene® nonliposomal transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid for 1 hour before being transferred to a media with 500 µL 15PPT ASW and incubated in an environment with light at 25°C.	24
22	*B. calyciflorus* resting eggs were transfected with Viromer® Yellow polymeric transfection solution and	24

	pCS2 plasmid (control) or pCS2eGFP plasmid for 1 hour before being transferred to a media with 500 µL spring water and incubated in an environment with light at 25°C.	
23	*B. calyciflorus* resting eggs were hydrated with Viromer® Yellow polymeric transfection solution and pCS2 plasmid (control) or pCS2eGFP plasmid and incubated in an environment with light at 25°C.	24
24	*B. manjavacas* hatchlings, resultant from experiment 21's transfection over a period of 24 hours, were subsequently transferred to an environment without light and incubated at 30°C for another 24 hours.	48
25	*B. manjavacas* hatchlings, resultant from experiment 22's transfection over a period of 24 hours, were subsequently transferred to an environment without light and incubated at 30°C for another 24 hours.	48
26	*B. calyciflorus* hatchlings, resultant from experiment 23's transfection over a period of 24 hours, were subsequently transferred to an environment without light and incubated at 30°C for another 24 hours.	48
27	Decapsulated *B. manjavacas* eggs, with no additional rehydration steps, were transfected in wells with 15 PPT ASW, Viromer® Yellow polymeric transfection solution, and pCS2 plasmid (control) or pCS2eGFP plasmid and subsequently incubated in an environment without light at 25°C.	24
28	*B. manjavacas* neonates were transfected in wells with 15 PPT ASW, Fugene® nonliposomal transfection solution, Viromer® Yellow polymeric transfection solution, and pCS2 plasmid (control) or pCS2eGFP plasmid and subsequently incubated in an environment without light at 25°C.	24
29	*B. manjavacas* neonates were transfected in wells with 15 PPT ASW, Viromer® Yellow polymeric transfection solution, and pCS2 plasmid (control) or pCS2eGFP plasmid and subsequently incubated in an environment without light at 25°C for 1 hour before being transferred to another environment without light at 5°C.	24
30	*B. manjavacas*, resultant from experiment 30's transfection over a period of 24 hours, were subsequently transferred to another environment without light and incubated at 22°C for another 24 hours.	48

Once the organisms had incubated for a period of 24-48 hours, transfection within a representative population of 20 – 50 organisms was verified through the presence of green fluorescent proteins under fluorescence microscopy. Transfection efficacy was measured as the percentage of animals displaying strong localized fluorescence under a GFP filter set at 200ms exposure.

CHAPTER 3

RESULTS

Lawn growth was observed both on the LB (no ampicillin) plate with the transformed plasmid containing the shRNA construct (Figure 2) as well as on the control LB (no ampicillin) plate with the unmodified pCS2eGFP plasmid vector (Figure 3).

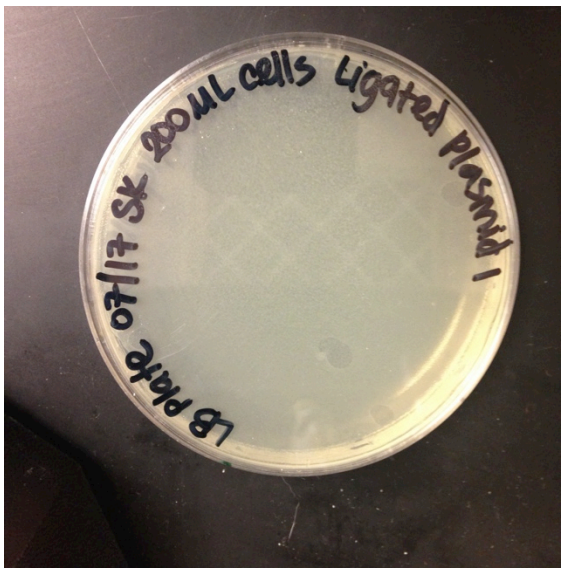

Figure 2: Presence of lawn growth in LB plate with the transformed ligated plasmid
Caption: Lawn growth was observed on LB plates that were plated with 200µL of the transformed pCS2eGFP plasmid with the shRNA insert ligated within the plasmid.

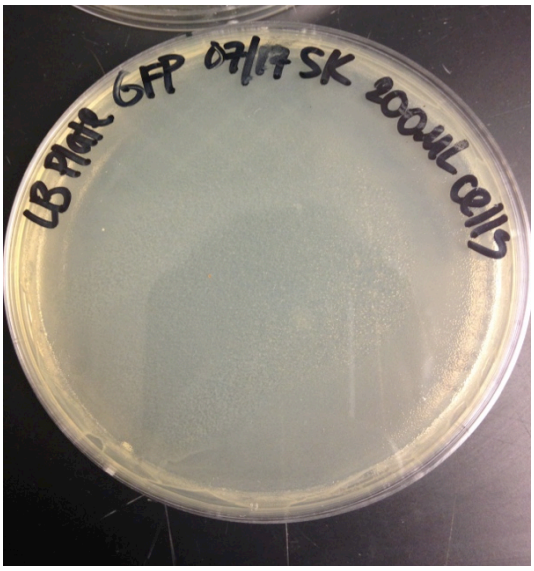

Figure 3: Presence of lawn growth in LB plate with the transformed control pCS2eGFP plasmid
Caption: Lawn growth was observed on LB plates that were plated with 200µL of the transformed unmodified control pCS2eGFP plasmid.

An abundance of colonies were observed both on the LB + Ampicillin plate with the 200µL of the transformed pCS2eGFP plasmid with the shRNA insert ligated within the plasmid and on the LB + Ampicillin plate with the 200µL of the transformed unmodified control pCS2eGFP plasmid vector (Figure 4).

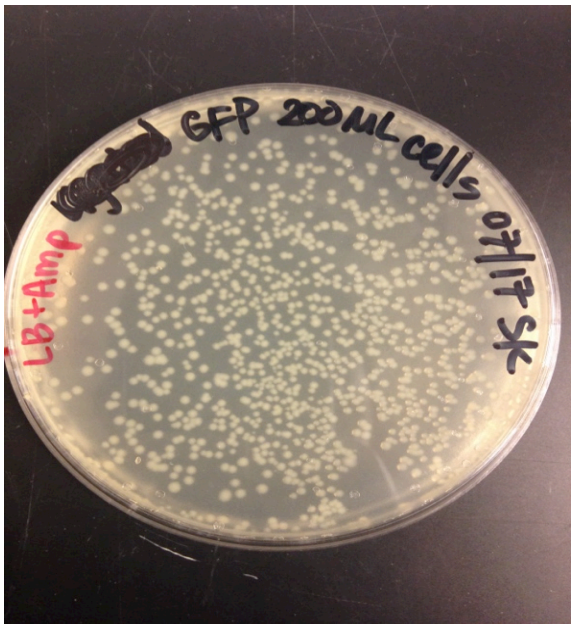

Figure 4: Presence of colony growth in LB + Ampicillin plate with the transformed control pCS2eGFP plasmid
Caption: An abundance of colonies were observed on LB + Ampicillin plates that were plated with 200µL of the transformed unmodified control pCS2eGFP plasmid.

Colony PCR was then conducted on the colonies collected from the LB + Ampicillin plates with the transformed unmodified control pCS2eGFP plasmid vector, using the SP6 forward primer and a reverse pCS2+ primer, designed from the unmodified pCS2eGFP plasmid, to verify for the insertion of the shRNA into the plasmid. An alignment of bands from both the positive control pCS2eGFP plasmid as well as the control plate colonies was observed, as demonstrated by the colony PCR results from Figure 5.

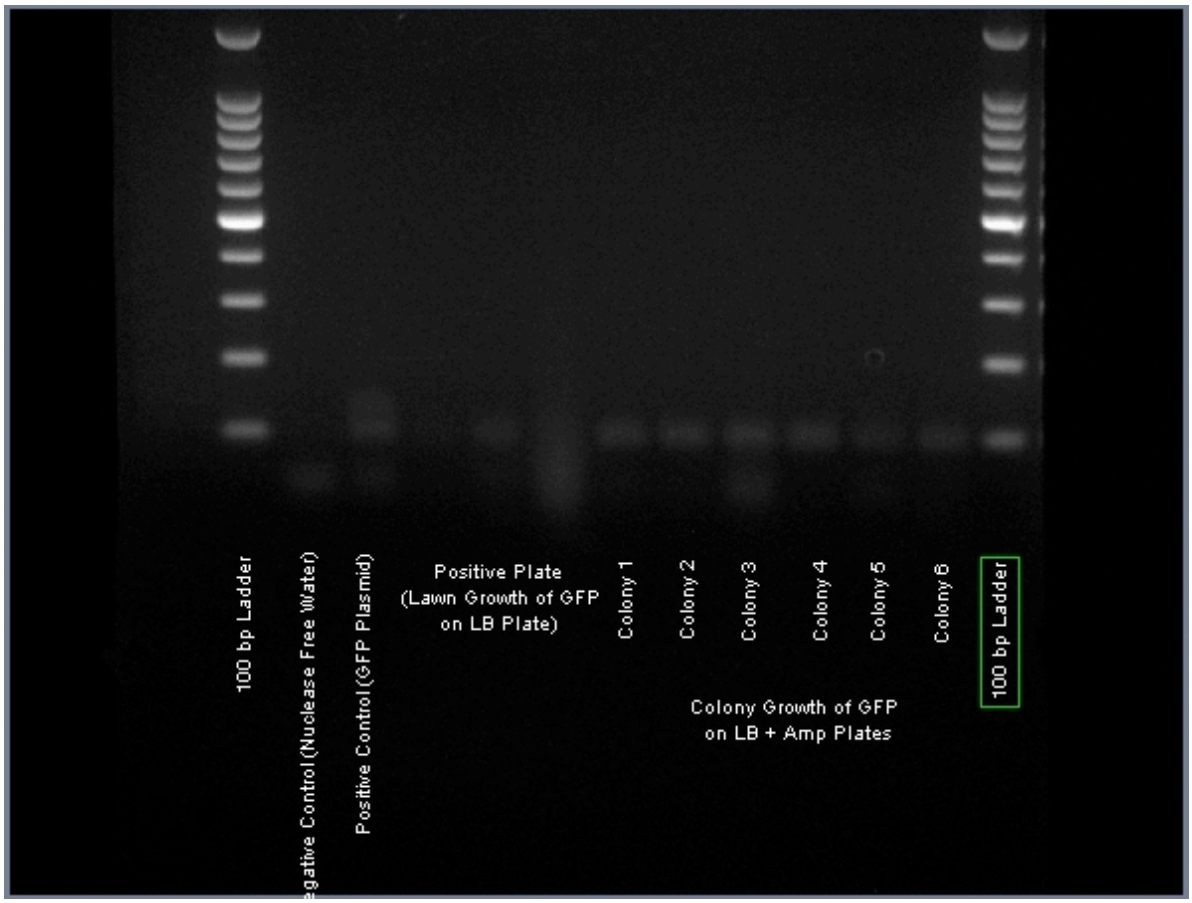

Figure 5: Colony PCR of colonies grown on the control LB and Ampicillin Plate with transformed unmodified control pCS2eGFP plasmid

Caption: An alignment of bands were observed through the administration of Colony PCR on the transformed unmodified control pCS2eGFP plasmid colonies and the positive control, the pCS2eGFP plasmid.

Colony PCR was also conducted on the colonies collected from the LB + Ampicillin plates with the transformed pCS2eGFP plasmid with the shRNA insert ligated within the plasmid, using the SP6 forward primer and a reverse pCS2+ primer, designed from the unmodified pCS2eGFP plasmid, to detect the insertion of the shRNA into the plasmid vector. An upward shift of bands was observed in several of the transformed ligated pCS2eGFP plasmid colonies, in comparison to the control colonies collected from the LB + Ampicillin plates with the transformed unmodified control pCS2eGFP plasmid vector (Figure 6).

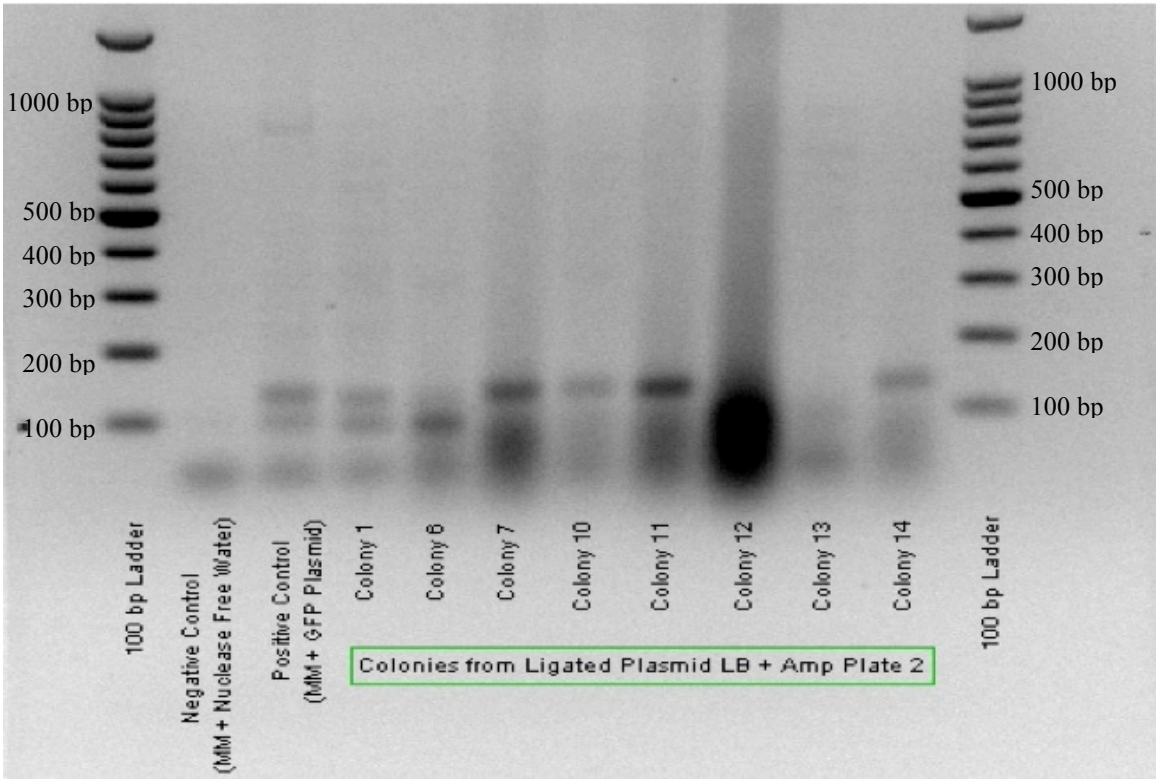

Figure 6: Colony PCR of colonies grown on the control LB and Ampicillin Plate with transformed pCS2eGFP plasmid with the shRNA insert ligated within the plasmid
Caption: Colony PCR of the transformed ligated plasmid colonies illustrate a 70 base pair upward shift of bands within the plasmid vector in colonies 7, 10, 11, and 14 when compared against unmodified pCS2eGFP positive control plasmid vector.

Colonies 7, 10, and 14 were subsequently Sanger sequenced, in order to verify for the presence of the shRNA insert within the plasmid vector. Colony 7's sequence illustrated a 100% exact match (Figure 7), and colonies 10 and 14 illustrated a near perfect match of gene sequences, in comparison to the expected pCS2eGFP-TRP7 sequence.

```
pCS2_TRP7_7       GGATCCGGTCCTTATTTGGTCTTAGTGAACACACTAAGACCAAATAAGGACCCCATGGTG
TRP7_Expected     -GATCCGGTCCTTATTTGGTCTTAGTGAACACACTAAGACCAAATAAGGACCC-------
                   ***************************************************
```

Figure 7: Sanger sequencing results of transformed ligated plasmid colony 7
Caption: Sanger sequencing revealed that Colony 7's sequence was an exact match in comparison to the expected pCS2eGFP-TRP7 sequence.

Upon verification of successful insertion and cloning of the TRP7 shRNA construct, an optimization of the transfection protocol was explored through multiple experiments on organisms from the species *B. manjavacas, B. rotundiformis, B. plicatilis,* and *B. calyciflorus*. A significant differential in localized fluorescence between the organisms transfected with the pCS2+ plasmid vector and organisms transfected with the pCS2eGFP plasmid vector with the GFP reporter gene was not observed in any treatment. A list of the results obtained from the varying transfection conditions examined (Table 2) is provided below.

Table 2: Results obtained from transfection experiments conducted on *B. manjavacas, B. rotundiformis, B. plicatilis,* and *B. calyciflorus*

Experiment	Percentage of Animals Exposed to the Transfection Reagent with No Plasmid Observed with Localized Green Fluorescence	Percentage of Animals Transfected with Control pCS2+ Plasmid Observed with Localized Green Fluorescence	Percentage of Animals Transfected with pCS2eGFP Plasmid Observed with Localized Green Fluorescence
1	0%	--	47%
2	--	12.50%*	4.50%*
3	--	0%	0%
4	--	5.56%*	0%
5	--	1.63%*	0%
6	--	0%	0%
7	--	0%	11.11%
8	--	0%	0%
9	--	0%	0%
10	--	0%	0%
11	--	0%	0%
12	--	0%	0%
13	--	0%	0%
14	--	0%	0%
15	--	0%	4.5%*
16	--	0%	0%
17	--	0%	0%
18	--	Animals never hatched from the resting eggs.	Animals never hatched from the resting eggs.
19	--	0%	0%
20	--	0%	0%
21	--	0%	0%
22	--	42.47%	25.83%
23	--	0%	0%
24	--	0%	0%
25	--	0%	0%
26	--	0%	0%
27	--	90.90%	81.25%
28	--	0%	0%
29	--	35.29%	15.63%
30	--	20%	3.92%

* Indicates that localized fluorescence, in the animals that illustrated localized green fluorescence, was observed exclusively within the mastax of the organisms.

CHAPTER 4

DISCUSSION

As illustrated by figures 2 and 3, lawn growth was observed both on the LB plate with the ligated plasmid as well as on the control LB plate with the unmodified pCS2eGFP plasmid. This indicates that the cells were not damaged or killed during the transformation process. The transformed ligated plasmid cells as well as the transformed unmodified pCS2eGFP plasmid cells were subsequently plated on plates that contained both LB and Ampicillin. The purpose of ampicillin, an antibiotic shown to inhibit growth in bacteria, in these plates is to inhibit growth of untransformed bacteria that do not express the gene for ampicillin resistance contained in the pCS2+ plasmid. The unmodified pCS2+ plasmid contains the ampicillin resistance gene, allowing for colonies of plasmids to grow despite the presence of ampicillin within the environment. This signifies that any colonies observed in the LB + Ampicillin plates with the transformed ligated plasmid cells or the transformed unmodified pCS2eGFP plasmid cells either have a ligated plasmid with the insert or the unmodified pCS2 plasmid, respectively.

The control plasmid plate demonstrated exceptional colony growth. The unaffected original pCS2eGFP plasmid contains the ampicillin resistance gene, so the colony growth observed in the control plasmid plate signifies that the transformation protocol functions efficiently. This is further confirmed through Colony PCR of the colonies from the plate. Colonies collected from the control plasmid plate were analyzed and compared against a positive control to verify if the transformation of the pCS2eGFP plasmid was successful as well as to observe if the plasmid contains the insert or not. 50

μg the unmodified, untransformed pCS2eGFP plasmid vector was used as the positive control. The resultant alignment of the bands from both the positive control as well as the control plate colonies, as illustrated by Figure 5, indicates that the transformation of the pCS2eGFP plasmid was successful. It also indicates the colonies do not contain a modified version of the pCS2eGFP plasmid with the insert due to the fact that the bands were all at an equivalent length. An upward shift would be expected if the plasmid colonies contained the insert, as that would make the plasmid contain more nucleotides, causing it to travel down the gel less than the unmodified plasmid without the insert. Such was observed in the Colony PCR of the colonies collected from the transformed ligated plasmid plate, as illustrated by Figure 6.

The ligated plasmid plate, like the control plasmid plate, demonstrated exceptional colony growth. This indicates that the insert was successfully ligated within the plasmid, thereby allowing for colony growth of the plasmid on the plates with the ampicillin. The vector, once again in its original circular form after the digestion of the plasmid and subsequent ligation of the shRNA insert within the plasmid vector, is able to express the ampicillin resistance gene within the pCS2+ plasmid, facilitating the growth of colonies of the modified ligated plasmid on the plates with ampicillin. Colony PCR of these resultant colonies, collected from the transformed ligated plasmid plate, detects an upward shift of bands in colonies 7, 10, 11, and 14, in comparison to the unmodified, untransformed pCS2eGFP plasmid vector positive control. This upward shift of bands, as observed in figure 6, indicates the presence of the shRNA insert within the plasmid, encouraging the use of Sanger sequencing for verification of the presence of the insert. Sanger sequencing illustrated a 100% genomic match between the expected ligated

plasmid sequence and the sequence obtained from colony 7 and a near perfect match between the expected ligated plasmid sequence and the sequence obtained from colonies 10 and 14. This detection and verification of the TRP shRNA construct within the pCS2eGFP plasmid suggests that a working protocol for plasmid cloning and shRNA design has been established.

With the establishment of a working protocol for plasmid cloning and shRNA design, the optimization of a transfection protocol for rotifers was explored. Rotifer resting eggs, decapsulated eggs, and hatchlings from rotifer species *B. manjavacas, B. plicatilis, B. rotundiformus,* and *B. calyciflorus* were transfected with various different transfection agents so to ascertain a transfection technique that yielded high rates of plasmid expression. The pCS2+ plasmid was transfected as the control, and the pCS2eGFP plasmid, illustrated in Figure 1, were transfected into rotifers, so the presence of green fluorescence, in response to the activation of the green fluorescent protein reporter gene, could be used as an indication of efficacious transfection of the plasmid vector within the rotifer. A significant differential in localized fluorescence between the organisms transfected with the pCS2+ plasmid vector and organisms transfected with the pCS2eGFP plasmid vector with the GFP reporter gene, however, was not observed. It is strongly speculated that this is due to the potential that though the plasmid may be transfected into the rotifers, the promoter is not being effectively expressed by the rotifer polymerases. Transfection of *B. manjavacas* with heat shock protein double stranded RNA has been observed to yield reduction in expression of its respective genes, indicating that it is plausible to transfect RNA with *B. manjavacas*[16]. As such, it is speculated that the cause of the absence of significant differential in localized

fluorescence between the organisms transfected with the pCS2+ plasmid vector and organisms transfected with the pCS2eGFP plasmid is due to absence of expression of the GFP. Alternative promoters will be explored within the various species of rotifers, so as to identify the optimal promoter region to verify for plasmid transfection within the rotifers.

In conclusion, with the establishment of a working protocol for plasmid cloning and shRNA design as a result of this study, gene knockdown in rotifers can be further explored. The ligated plasmid with the shRNA insert of interest can be harvested from transformed bacteria to use for gene knockdown studies in rotifers, and genes can be manipulated through plasmid vector insertions, thereby inducing the silencing of the gene's expression with shRNA, via RNAi, within the rotifers. Transfected rotifers can then be further analyzed and examined for the biological effects such as changes in fecundity and lifespan. Additionally, with demonstration of RNAi knockdown in the known gene, optimization of the transfection protocol in rotifers can also be explored. With increased efficiency in the transfection of rotifers, populations of rotifers expressing the plasmid can be amassed, allowing for experimental design that examine the varying aging mechanisms and effects that are stimulated due to permanent changes in target gene expression. Additional gene pathways and mechanisms speculated to cause an increase in average lifespan could be further explored and investigated. Those results could lead to the identification of evolutionarily conserved genes that regulate organismal aging, which could lead to further implications in the field of pharmacological intervention in mammalian aging as well as in the field of biogerontology overall.

REFERENCES

[1] Li, F., & Mahato, R. I. (2011). RNA interference for improving the outcome of islet transplantation. *Advanced Drug Delivery Reviews*.doi:10.1016/j.addr.2010.11.003

[2] Sander, M., Oxlund, B., Jespersen, A., Krasnik, A., Mortensen, E., Westendorp, R., & Rasmussen, L. (2014). The challenges of human population ageing. Age and Ageing, 185-187.

[3] Bartke A. Insulin and aging. Cell Cycle. 2008;7:3338–3343.

[4] Smith, E., Tsuchiya, M., Fox, L., Dang, N., Hu, D., Kerr, E., . . . Kennedy, B. (2008). Quantitative evidence for conserved longevity pathways between divergent eukaryotic species. *Genome Research,* 564-570.

[5] Kortschak, R. (2003). EST Analysis of the Cnidarian Acropora millepora Reveals Extensive Gene Loss and Rapid Sequence Divergence in the Model Invertebrates. *Current Biology,* 2190-2195.

[6] Austad SN. Is there a role for new invertebrate models for aging research? - J Gerontol A Biol Sci Med Sci. 2009;64A:192–14.

[7] Snell T. Rotifers as models for the biology of aging. Int Rev Hydrobiol. Mar 2014; 99(1-2): 84–95.

[8] Eisenmann, D. M., Wnt signaling (June 25, 2005), *WormBook*, ed. The *C. elegans* Research Community, WormBook, doi/10.1895/wormbook.1.7.1, http://www.wormbook.org.

[9] Taxman, D., Moore, C., Guthrie, E., & Huang, M. (n.d.). Short Hairpin RNA (shRNA): Design, Delivery, and Assessment of Gene Knockdown. *Methods in Molecular Biology RNA Therapeutics,* 139-156.

[10] Ihle, K., Fondrk, M., Page, R., & Amdam, G. (n.d.). Genotype effect on lifespan following vitellogenin knockdown. *Experimental Gerontology,* 113-122.

[11] Shearer, T., & Snell, T. (2007). Transfection of siRNA into Brachionus plicatilis (Rotifera). *Hydrobiologia,* 141-150.

[12] Liu, B., Liou, J., Chen, Y., Huang, Y., & Lee, H. (2013). Delivery of Nucleic Acids, Proteins, and Nanoparticles by Arginine-Rich Cell-Penetrating Peptides in Rotifers. *Marine Biotechnology Mar Biotechnol,* 584-595.

[13] Johnston, R. K., & Snell, T. W. (2016). Moderately lower temperatures greatly extend the lifespan of Brachionus manjavacas (Rotifera): Thermodynamics or gene regulation? Experimental Gerontology, 78, 12-22. doi:10.1016/j.exger.2016.02.014

[14] Nilius, B., & Flockerzi, V. (2014). *Mammalian Transient Receptor Potential (TRP) Cation Channels Volume I*. Berlin, Heidelberg: Springer.

[15] Kim, G., Moon, J., Han, J., Kim, K., & Rhim, H. (2011). The sCMV IE enhancer/promoter system for high-level expression and efficient functional studies of target genes in mammalian cells and zebrafish. *Biotechnology Letters Biotechnol Lett,* 19-1326.

[16] Smith, H., Burns, A., Shearer, T., & Snell, T. (2011). Three heat shock proteins are essential for rotifer thermotolerance. *Journal of Experimental Marine Biology and Ecology,* 1-6. Retrieved December 2, 2015.